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Rutin activates the MAPK pathway and BDNF gene expression on beta-amyloid induced neurotoxicity in rats

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HIGHLIGHTS

- The effect of rutin on memory was evaluated in rats injected with Aβ.
- Rutin increased ERK1, CREB and BDNF gene expression in the hippocampus.
- Rutin increased memory retrieval in passive avoidance task.
- Rutin decreased oxidative stress of Aβ on memory.
- Rutin potentially ameliorates the destructive effects of Aβ on memory.

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ABSTRACT

Flavonoids are present in foods such as fruits and vegetables. A relationship between the consumption of flavonoid-rich foods and prevention of human disease including neurodegenerative disorders has been demonstrated. We assessed the effect of rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) on the mitogen-activated protein kinase (MAPK) pathway, memory retrieval and oxidative stress in rats injected with β-amyloid (Aβ), which is implicated to have an important role in Alzheimer's disease (AD). Aβ was injected bilaterally in the deep frontal cortex of rat brain. Next, rutin and saline were injected (i.p.) for 3 weeks. In comparison to the control group, rutin significantly increased extracellular signal-regulated protein kinase 1 (ERK1), cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) gene expression in the hippocampus of rats. Rutin (100 mg/kg) significantly increased memory retrieval compared to the control group. Malondialdehyde (MDA) level in the hippocampus of the rutin group was significantly lower than those in the control group. The content of sulfhydryl groups in the rutin group was higher than that in the control group. The findings show a possibility that rutin may have beneficial effects against neurotoxicity of Aβ on memory in rats.

Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; Akt/PKB, protein kinase B; Arc, activity-regulated cytoskeleton-associated protein; BDNF, brain-derived neurotrophic factor; CAT, catalase; CREB, cAMP response element-binding protein; GSH, glutathione; GPx, glutathione peroxidase; ERK1/2, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; i.p., interaperitoneally; MAP kinase, mitogen-activated protein kinase; MCAO, middle cerebral artery occlusion; MEK1/2, mitogen activated kinase kinase; MDA, malondialdehyde; mTor, mammalian target of rapamycin; PBS, phosphate buffered saline; PI3K, phosphatidyl inositiol 3-kinase; PTZ, pentylenetetrazole; SH, sulfhydryl; SOD, superoxide dismutase; TrkB, tropomyosin receptor kinase B; ROS, reactive oxygen species.

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1. Introduction

Alzheimer disease (AD) is the most common cause of dementia. Its prevalence ranges from 3% to almost 50% between the ages of 65–85 years (Simon et al., 2009). In recent years, flavonoid derivatives have been proposed to be useful in the treatment of neurodegenerative disorders such as AD (Vauzour et al., 2008; Patel et al., 2008). Flavonoids such as puerarin, baicalein and kaempferol have been reported to be protective against Aβ peptide neurotoxicity in *in vitro* and *in vivo* studies (Kim et al., 2010; Lu et al., 2011; Zhang et al., 2008). Flavonoids are polyphenolic compounds found in food plants, and are divided into the following six groups: flavonols, flavones, isoflavone, flavanones, flavanols and anthocyanidins (Spencer, 2008).

Rutin (quercetin-3-O-rutinoside) is a flavonol found in plants such as buckwheat, passion flower, apple and tea (Gulpinar et al., 2012; Kuntić et al., 2007). Rutin has been found to have several neuropharmacological effects including anticonvulsant, antidepressant and neuroprotective effects on the central nervous system (Khan et al., 2009; Machado et al., 2008; Nassiri-Asl et al., 2008; Tongjaroenbuangam et al., 2011; Yang et al., 2012). Several studies have also focused on the effects of rutin on cognition and memory in different models of memory impairment in different animals (Gupta et al., 2003; Pu et al., 2007; Pyrzanowska et al., 2012; Richetti et al., 2011).

Previously, we have found that rutin has a potential role in enhancing memory retrieval. We observed that 1 week of daily administration of rutin (10 mg/kg) prior to training resulted in increased retrieval of memory in the first, second and third retention tests of passive avoidance test in normal rats (Nassiri-Asl et al., 2010). Furthermore, pretreatment with rutin (50–100 mg/kg) before administration of pentylenetetrazole (PTZ) every other day prior to training significantly decreased the severity of seizures and resulted in increased retrieval of memory in kindled rats during the first and second retention tests of the passive avoidance task (Nassiri-Asl et al., 2010). Recently, it was reported that rutin supplementation was effective in suppressing memory dysfunction caused by streptozotocin in rats. It reduced inflammation and prevented the morphological changes induced by streptozotocin in the hippocampus (Javed et al., 2012).

The mitogen-activated protein kinase (MAPK) cascade that includes extracellular signal-regulated protein kinase (ERK1/2) and cAMP response element-binding protein (CREB) is involved in neural survival and plasticity. It has been shown that activation of this cascade causes long lasting changes in synaptic plasticity and memory (Spencer, 2008). BDNF is a neurotrophin that affects the survival and function of neurons in the central nervous system and is important for appropriate synaptic connection formation during development and for learning and memory in adults (Thomas and Davies, 2005).

It has been suggested that the Aβ, the major protein component of senile plaque, plays an important role in pathogenesis of AD. The Aβ-related fragments (Aβ (1–40), Aβ (1–42)) exhibit toxicity to neurons (Shin et al., 1997; Wang et al., 2001). Aβ aggregation could contribute to memory impairment especially through inducing transcripts of the genes involved in inflammatory or apoptotic pathways (Hemmati et al., 2013).

Thus, in this study, we focused on the possible effects of rutin on MAPK and BDNF gene expression and memory retrieval in β-amyloid-injected rats. Furthermore, malondialdehyde (MDA) and sulfhydryl (SH) groups were measured as indicators of lipid peroxidation and oxidative stress in this model of memory impairment.

2. Materials and methods

2.1. Animals

Male Wistar rats (200–250 g) were obtained from the Razi Institute (Karaj, Iran) and housed in groups of four per cage under standard laboratory conditions. Rats were housed in a room maintained at a constant room temperature (21 ± 2 °C) under a 12L:12D cycle with free access to food and water. All animal experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) to minimize the number of animals used and their suffering.

2.2. Chemicals

Rutin, β-amyloid (Aβ) (1–42) and phosphate buffered saline (PBS) were purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Other drugs used in this study included xylazine (Loughrea, Co. Galway, Ireland) and ketamine (Rotexmedica, GmbH, Germany). 2,2′-Dinitro-5,5′-dithiodibenzoic acid (DTNB), 2-thiobarbituric acid (TBA), Tris (Trizma® base), sodium ethylenediaminetetraacetic acid (Na EDTA), methanol, trichloroacetic acid (TCA), potassium chloride (KCl) and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). Rutin daily dissolved in saline at concentration 220 mg/5 ml and was injected i.p. to animals.

2.3. Preparation of Aβ and surgery

Aβ (1–42) fragments were prepared as stock solutions in sterile 0.1 M PBS (pH 7.4) and aliquoted and stored at −80 °C until use (van der Stelt et al., 2006). After defrosting the aliquots, 3 μl of Aβ solution (30 ng) was used for each injection.

2.4. Surgery and experimental procedures

The rats were anaesthetized with ketamine (60 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Aβ was bilaterally injected into the animal's frontal cortex (Hemmati et al., 2013) as follows: anterior–posterior (AP), 3.2 mm from the bregma; medial–lateral (ML), 2 mm from the midline and dorsal–ventral (DV), 3 mm from the skull surface (Paxinos and Watson, 1998).

For administration of Aβ, animals were gently hand-restrained and drug infusions were made using an injection needle (24-gauge) connected through a polyethylene tube to a 5 μl Hamilton syringe (infusion rate 1 ml/min). The needles were left in place for 1 min following the microinjections to minimize the flow of drug solution up the track (Nassiri-Asl et al., 2008). Rats were divided into two groups of 30 animals each. Aβ was injected bilaterally (3 μl = 30 ng each side) in the deep frontal cortex of the rat brain. In the control group, saline (10 ml/kg, i.p.) was injected after administration of Aβ, every day for 3 weeks. In second group, rutin (100 mg/kg, i.p.) was injected after administration of Aβ, every day for 3 weeks.

2.5. Passive avoidance apparatus

After 3 weeks of injection of Aβ and treatment of animals with saline and rutin, the passive avoidance task was conducted in animals. All rats were allowed to habituate, and the acquisition trial was performed 30 min after the habituation trial. Then, each rat was gently placed in the light compartment of the apparatus. After 5 s, the guillotine door was opened and the rat was allowed to enter the dark compartment. The latency with which each rat crossed into the dark compartment was recorded. The rats that waited more than 100 s to cross into the dark compartment were eliminated from the experiments. Once the rat crossed with all four paws into the next compartment, the guillotine door was closed and the rat was returned to its home cage. The acquisition trial was performed 30 min after the habituation trial. The rat was placed in the light compartment, and the guillotine door was opened 5 s later. As soon as the rat crossed into the dark compartment, the door was closed and a foot shock (0.5 mA intensity, 3 s) was immediately delivered to the grid floor of the dark room by an insulated stimulator (Nassiri-Asl et al., 2012).

One day after training, retention tests were conducted to evaluate memory performance. Each rat was placed in the light compartment for 20 s, the door was opened, and the step-through latency for entering into the dark compartment was measured. The test session ended when the rat entered the dark compartment or remained in the light compartment for 300 s. No electric shock was applied during these sessions (Nassiri-Asl et al., 2012).

At the end of the behavioral studies, rats were anaesthetized with i.p. injection of ketamine/xylazine (60 mg/kg and 6 mg/kg, respectively). Animals were sacrificed under anesthesia. Then, whole hippocampal tissues were dissected and quickly removed and cleaned with chilled saline. One hippocampus of each brain was used for biochemical analysis and the other hippocampus was snap frozen and kept at −80 °C to be later used for gene expression analysis (Hemmati et al., 2013).

Table 1
The oligonucleotide primers used in real time PCR assay.

Target and internal control genes	Sequence	Amplicon size (bp)
BDNF	F: CGGCTTGGAGAAGGAAAC R: GAACCCGGTCTCATCAAAG	71
ERK1	F: GTTCTGGAATGGAAGGGCTA R: GGATGAGTAGGGCAGAGCTT	91
ERK2	F: CAGTTCTTGACCCTGGTCCT R: AACGGCTCAAAGGAGTCAAG	68
CREB1	F: TACAGGGCCTGCAGACATTA R: TTGCTGGGCACTAGAATCTG	112
β-Actin	F: AGCGCAAGTACTCTGTGTGG R: AACAGTCCGCCTAGAAGCAT	138

2.6. Real-time PCR and comparative threshold cycle method

Then, frozen whole hippocampus tissues were homogenated using an Ultrasonic Processor UP100H (Hielsher, Germany). Total RNA was extracted using the total RNA extraction kit (Jena Bioscience, GmbH, Jena, Germany). Next, RNA was reverse transcribed using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, Waltham, MA, USA).

Quantitative RT-PCR was used to detect BDNF, ERK1, ERK2 and CREB1 RNA content in hippocampal tissues. The target and β-actin (internal control) genes were amplified with appropriate primers (Table 1). All primers were designed using Gene Runner software (version 3.05). SYBR Green I real-time PCR assay was performed in final reaction volumes of 20 μl with 10 μl of SYBR Green I Master Mix (Bioneer, Korea), 10 pmol of forward and reverse primers and 20 ng total RNA-derived cDNAs. Thermal cycling was performed using the ABI-7500 (Applied Biosystems, Foster, CA, USA) Sequence Detection System using the following cycling condition: 10 min at 95 °C for the first denaturation step, followed by 40 cycles at 95 °C for 20 s and 55 °C for 45 s. Each complete amplification stage was followed by dissociation stage; at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The $2^{-\Delta CT}$ method of relative quantification was used to determine the fold change in expression. This was done by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control (β-actin) in the treated and untreated samples ($\Delta CT = CT_{target} - CT_{\beta\text{-actin}}$) (Schmittgen and Livak, 2008).

2.7. Measurement of lipid peroxidation

MDA reacts with TBA as a thiobarbituric acid reactive substance (TBARS) to produce a red complex that has a peak absorbance at 535 nm (Janero, 1990). Hippocampi were homogenized with cold 1.5% KCl to make a 10% homogenate. To 1.0 ml of the brain homogenate, 2.0 ml of TCA–TBA–HCl was added and mixed thoroughly. The solution was heated for 60 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 × *g* for 10 min. The absorbance was determined at 535 nm against a blank that contained all the reagents except the sample. The amount of MDA equivalents formed was calculated using a molar extinction coefficient of 1.56×10^5 mol^{-1} cm^{-1} and expressed as nmol MDA equivalents/mg protein.

2.8. Measurement of total SH groups

Total SH groups were measured using DTNB. This reagent reacts with the SH groups to produce a yellow complex that has a peak absorbance at 412 nm (Ellman, 1959). Briefly, 1 ml Tris–EDTA buffer (pH = 8.6) was added to 50 μl hippocampal homogenate in 2-ml cuvettes and sample absorbance was read at 412 nm against Tris–EDTA buffer alone (A_1). Next, 20 μl of DTNB (10 mM in methanol) was added to the mixture, and after 15 min (stored at laboratory temperature), the sample absorbance was read again (A_2). The absorbance of the DTNB reagent was read as a blank (B) (Hosseinzadeh et al., 2012). Total thiol concentration (mM) was calculated from the following equation: total thiol concentration (mM) = $(A_2 - A_1 - B) \times (1.07/0.05) \times 13.6$.

2.9. Data analysis

Data were expressed as mean ± standard error of the mean (S.E.M.) using SPSS (version 20). Data from behavioral studies, oxidative stress and gene expression were analyzed using independent-sample *T*-tests with a confidence level of 95%. The normality distribution of samples with the Kolmogorov–Smirnov test was done ($P > 0.05$). $P < 0.05$ was considered to be statistically significant.

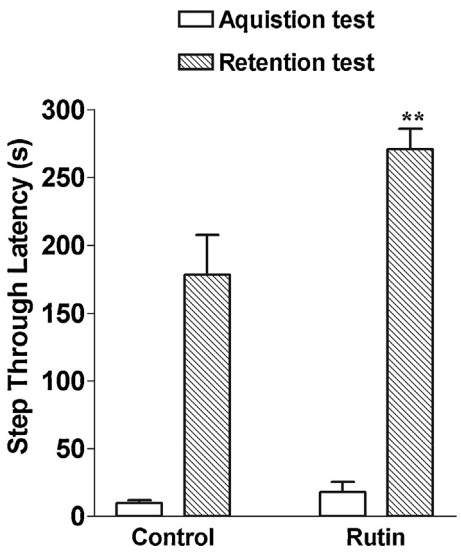

Fig. 1. The effects of 100 mg/kg rutin on the acquisition and retention tests in β-amyloid-injected rats. After 3 weeks of administration of Aβ and treatment of animals with saline and rutin (100 mg/kg), acquisition and retention tests were performed to evaluate memory performance. Data are mean ± SEM, $^{**}P < 0.01$ compared to control group, $n = 10$.

3. Results

3.1. Effects of rutin on passive avoidance on Aβ-induced neurotoxicity

There was no significant difference between the different groups in the number of trials, thus confirming the uniformity of the groups. All animals reached the criteria during the training procedure. In the control and rutin groups, the acquisition trials and memory retrieval were measured as shown in Fig. 1.

Rutin at a dose of 100 mg/kg significantly increased memory retrieval compared to the control group ($t_{(21)} = 2.859$, $P < 0.01$) (Fig. 1). Also, there were no significant differences in the acquisition trials between the two groups ($t_{(19)} = 0.976$, $P = 0.34$).

3.2. Effects of rutin on ERK1, ERK2, CREB and BDNF gene expression

Rutin significantly increased ERK1, CREB and BDNF gene expression in the hippocampi of rats compared to controls ($t_{(20)} = 2.52$,

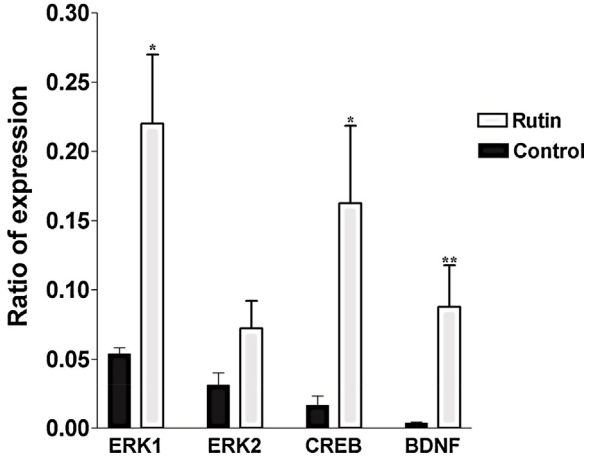

Fig. 2. Effects of rutin on CREB, ERK1, ERK2 and BDNF gene expression in the hippocampi of rats injected with β-amyloid. Data are mean ± SEM, $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to control group, $n = 10$.

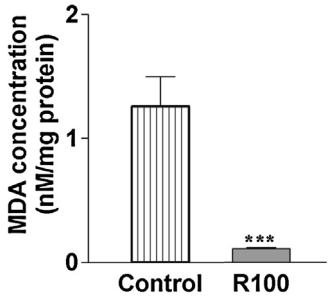

Fig. 3. Effect of rutin on MDA levels in the hippocampi of β-amyloid-injected rats. Values are mean ± SEM. ***$P < 0.001$ compared to control group, $n = 10$.

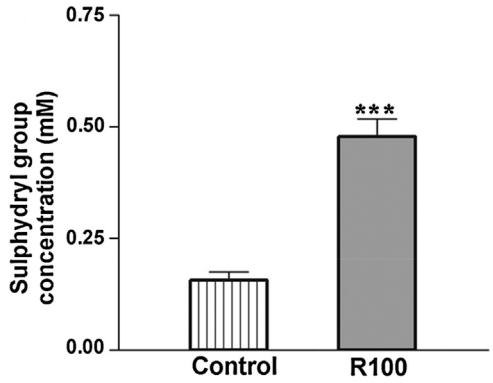

Fig. 4. Effect of rutin on total thiol concentrations in the hippocampus on β-amyloid-injected rats. Values are mean ± SEM. ***$P < 0.001$ compared to control group, $n = 10$.

$P = 0.02$, $t_{(17)} = 2.172$, $P = 0.04$, $t_{(11)} = 2.762$, $P = 0.01$) (Fig. 2). However, ERK2 expression level in the rutin group was not significantly different from that in the control group ($t_{(17)} = 1.162$, $P = 0.125$) (Fig. 2).

3.3. TBARS measurement

Post-treatment with rutin 100 mg/kg significantly decreased free radical-mediated lipid peroxidation compared to that in the control group ($t_{(21)} = 6.486$, $P < 0.001$) (Fig. 3).

3.4. Total SH groups assay

The average content of SH groups in the hippocampi of rats administered with 100 mg/kg rutin was significantly higher than that in the hippocampi of the control group ($t_{(23)} = 14.212$, $P < 0.001$) (Fig. 4).

4. Discussion

In the present study, we investigated the effects of rutin against neurotoxicity of Aβ in rats. Rutin (100 mg/kg) significantly increased memory retrieval compared to the control group. It could significantly increase ERK1, CREB and BDNF gene expression in the hippocampi of rats compared to that in controls. MDA levels in the hippocampi of the rutin group were significantly lower than those in the control group. Furthermore, the content of SH groups in rutin group was higher than that in the control.

Sublethal concentration of Aβ selectively down-regulated BDNF signaling by inhibiting the activation of the Ras–MAPK/ERK and PI3K/Akt pathways and activation of critical transcription factors, such as CREB in cultured cortical neurons (Tong et al., 2004).

The oligomers of Aβ could inhibit active ERK and CREB in primary neurons and reduce the downstream post-synaptic protein NMDA receptor subunit (Ma et al., 2007). However, stage-dependent abnormalities have been shown for ERK in mRNA and protein expression in AD and AD model (Dineley et al., 2001; Webster et al., 2006).

The critical role of CREB has established in memory, both long-term consolidation and indirect regulation of short-term memory. Agents that enhance the activity of CREB have been suggested to facilitate memory consolidation through increasing gene expression that is important for long-term memory (Williams and Spencer, 2012). Reduced phosphorylation of CREB has been observed in post-mortem brains of AD patients. It seems that impaired CREB phosphorylation is involved in AD pathophysiology (Scott Bitner, 2012). It was shown that flavonoids activate the ERK–CREB pathway and activation of this cascade leads to increased expression and release of BDNF from the synapse through enhanced CREB activation (Spencer et al., 2009; Vauzour et al., 2008). BDNF levels are reduced in AD and this has been shown to correlate with loss of cognition function (Peng et al., 2005, 2009). It was also shown that BDNF binds to its pre- and post-synaptic receptors tropomyosin receptor kinase B (TrkB) and triggers glutamate release and phosphatidyl inositiol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) signaling and activity-regulated cytoskeleton-associated protein (Arc) synthesis leading to altered synaptic plasticity (Spencer, 2008).

As our results, it is possible that rutin as a flavonoid, could activate the ERK–CREB pathway and activation of this cascade leads to increased expression and release of BDNF from the synapse through enhanced CREB activation. Thus, it seems that up-regulation of ERK1, CREB and BDNF expression by rutin could improve memory and defend against of Aβ neurotoxicity.

Similar to our study, blueberries that contain anthocyanins and flavanols, enhanced spatial memory in old rats, which also exhibited increased CREB activity and BDNF (Williams et al., 2008). Furthermore, apigenin could ameliorate AD-associated learning and memory impairment via inhibiting oxidative stress, and restoring ERK/CREB/BDNF pathway in cerebral cortex of APP/PS1 mice (Zhao et al., 2013).

Furthermore, it was reported that rutin increased the viability of trunk neural crest cells in culture. However, cell differentiation and proliferation did not change. But, in the presence of mitogen activated kinase kinase (MEK1/2) inhibitors PD98059 (PD) or phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (LY) with rutin, the cell viability was inhibited. Thus, it was suggested that the effects of rutin may be mediated by the ERK and PI3K pathways (Nones et al., 2012). Low concentrations of quercetin (<10 μM), an oral metabolite of rutin, could also activate the MAP kinase pathway including ERK2, JNK1 and p38 leading to expression of some survival and defensive genes such as c-Fos, c-Jun, phase II detoxifying enzymes, glutathione S-transferase and quinone reductase, resulting in increased Akt and CREB phosphorylation and activated survival and protective mechanisms (Spencer et al., 2003).

Rutin showed anti-amyloidogenic effects *in vitro* by reversibly binding to the amyloid fibril structure of Aβ oligomer and monomers. Also, it reduced ROS generation in H_2O_2-treated APP-swe cells (Jiménez-Aliaga et al., 2011). It was suggested that free catechol moiety of ruin helps that father molecule to be oxidized to its ortho-quinone and this new compound provides metal chelator properties (Omololu et al., 2011). In addition, rutin dose dependently inhibited aggregation and cytotoxicity of Aβ, attenuated oxidative stress and decreased the production of nitric oxide and proinflammatory cytokines in SH-SY5Y neuroblastoma cells. It was suggested that the reduction of ROS production of rutin is related to the enhancement of superoxide dismutase (SOD), glutathione peroxidase (GPx) or catalase (CAT) activity and its inhibitory activity

on xanthine oxidase which is an important enzyme in the oxidative injury to tissue (Wang et al., 2012).

It was suggested that Aβ causes neurotoxicity via oxidative stress and increased lipid peroxidation in hippocampus. Similarly, it was reported that epicatechin could decrease lipid peroxidation and ROS and result in improved memory skills (Cuevas et al., 2009). Furthermore, pretreatment with rutin before global cerebral ischemia, improved impairment in short-term memory and motor coordination on ischemia–reperfusion-induced cerebral injury. Also, rutin decreased mitochondrial TBARS (Gupta et al., 2003). Also, rutin supplementation was effective in memory dysfunction induced by streptozotocin in Morris water maze test. It has also reduced TBARS, glutathione (GSH) and nitrite level (Javed et al., 2012).

Rutin has neuroprotective effect in the brain ischemia and pretreatment with rutin reduced TBARS, H_2O_2 and GSH in the hippocampus and frontal cortex in the middle cerebral artery occlusion (MCAO) in rats. It also ameliorated morphological damage and attenuated ischemic neural apoptosis by reducing the expression of p53 and increasing of antioxidant enzymatic activities (Khan et al., 2009).

Thus, it is possible that post-treatment of rutin not only activated the MAPK pathway but also inhibited oxidative stress and increased antioxidant activity during initiation of neurotoxicity induced by Aβ, and then, could reduce memory impairment in animals.

In conclusion, our finding showed a possibility that rutin may have beneficial effects against Aβ (1–42)-induced neurotoxicity. It improved memory impairment caused by injection of Aβ in rats through activation of MAPK and BDNF. Furthermore, it reduced oxidative stress in the hippocampi of rats by reducing MDA level and increasing thiol content in the hippocampus. Further studies are necessary to clarify the effects and molecular mechanisms of this flavonoid.

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